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Award Number: W81XWH-11-1-0001

TITLE: Identification of Novel Therapeutic Targets for Triple-Negative Breast Cancer

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REPORT DATE: December 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
December 2012	Annual Summary	01 December 2011–30 November 2012

4. TITLE AND SUBTITLE
Identification of Novel Therapeutic Targets for Triple-Negative Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER- W81XWH-11-1-0001

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Christine Shiang

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas M.D. Anderson Cancer Center
Houston, TX 77030-4009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This project aims to identify, validate, and characterize novel therapeutic targets for triple-negative breast cancer (TNBC). During the research period, the most significant finding was the activation of lipoprotein receptor related protein 8 (LRP8) and very low density lipoprotein receptor (VLDLR) signaling in triple-negative breast cancer which results in activation of glycolytic and lipid metabolism to drive TNBC tumor cell growth. Previously, the two membrane receptors were found to be highly overexpressed in human TNBC tumors and identified as the top-tier candidate targets from a custom high-throughput siRNA loss-of-function screen in 18 breast cancer cell lines. Specifically, the most significant finding during the report period was apolipoprotein E isoform 4 (ApoE4) mediated tumor cell stimulation and growth. In LRP8- and VLDLR-expressing breast cancer cell lines, ApoE4 stimulates cells in nutrient-poor conditions to allow them to proliferate through glycolytic and fatty acid synthesis pathways. In shLRP8 MDA-MB-231 xenografts, preliminary results show that tumor volume was significantly reduced and tumor cell necrosis was significantly higher than xenografts established by parental or shCON cells. The results suggest that LRP8 – APOE signaling may be an important therapeutic target for TNBC.

15. SUBJECT TERMS
Therapeutic targets, triple-negative breast cancer, cancer metabolism.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	U U	14	19b. TELEPHONE NUMBER *(include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Triple-negative breast cancers lack the expression of estrogen and progesterone receptors and human epidermal growth factor receptors. As such, the treatment for these cancers is restricted to cytotoxic chemotherapy, which kills both normal and cancerous cells. The purpose and scope of this project is to identify and validate new therapeutic targets for the specific and tailored treatment of triple-negative breast cancer.

BODY

The accompanying results report on tasks 5-9. The results from tasks 1-4 were reported in the 2011 Annual Summary.

Task 1	Months 1	Completed
Task 2	Months 1-2	Completed
Task 3	Months 3-12	Completed
Task 4	Months 13-15	Completed
Task 5	Months 16-18	Completed
Task 6	Months 15-18	Completed
Task 7	Months 19	Completed
Task 8	Months 20-21	Completed
Task 9	Months 22-24	In progress

Specific Aim 2: To determine the role of critical growth genes in the aggressive phenotype of triple-negative breast cancer cell lines.

Task 5: The effect of target gene knockdown on the function of TNBC cell lines will be assessed for the top druggable genes.

Results: Previously, exposure to reelin and ApoE isoform 4, ligands to LRP8 and VLDLR, stimulates the growth of ER-negative cells in vitro. The rate of cellular division was significantly increased, as evidenced in bromodeoxyuridine (BrdU) incorporation assays (Figure 1). siLRP8 treatment abrogated this response, indicating that the stimulatory effect was centered around LRP8 – ApoE4 signaling. The rate of apoptosis was not significantly reduced with ApoE4 or siLRP8 treatment, indicating that proliferation was the functional endpoint for LRP8- and VLDLR-expressing TNBC cells which are stimulated by ApoE4. We also examined to effect of a mouse monoclonal antibody against LRP8 reduced the binding affinity of recombinant human LRP8 and ApoE4 using an ELISA binding assay (Figure 2). In comparison, a mouse polyclonal antibody against the intracellular C-terminal region of LRP8 and mouse IgG antibody did not affect the binding of recombinant LRP8 and ApoE4 (Figure 2).

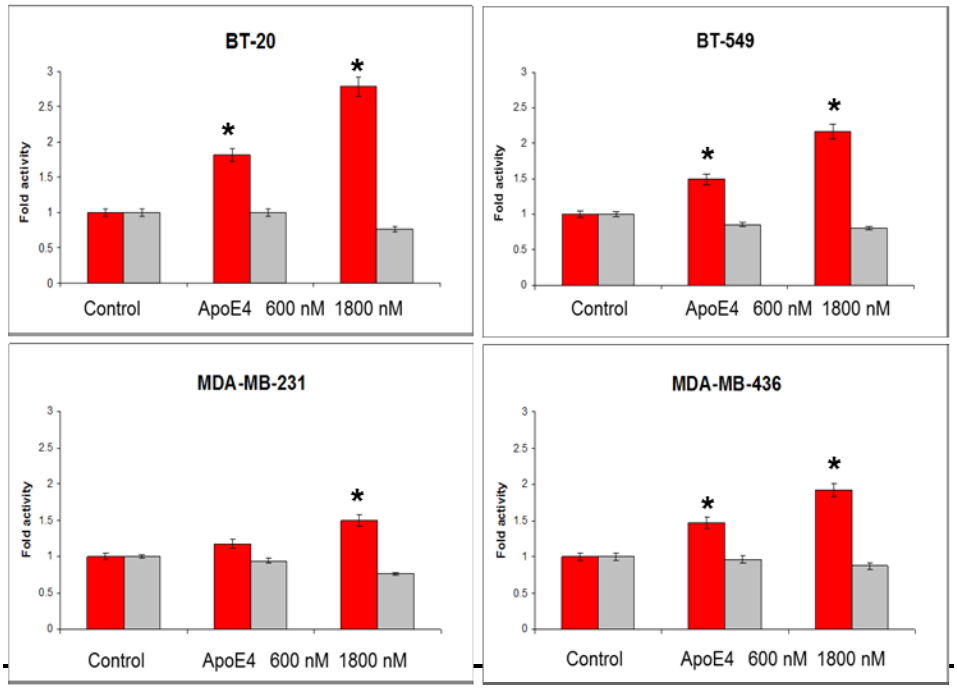

Figure 1. ApoE4 increased BrdU incorporation (shown in red) after 48 hours of treatment, increasing in activity from 600 to 1800 nM of ApoE4. Caspase 3, 7 activity (shown in gray) did not significantly change.

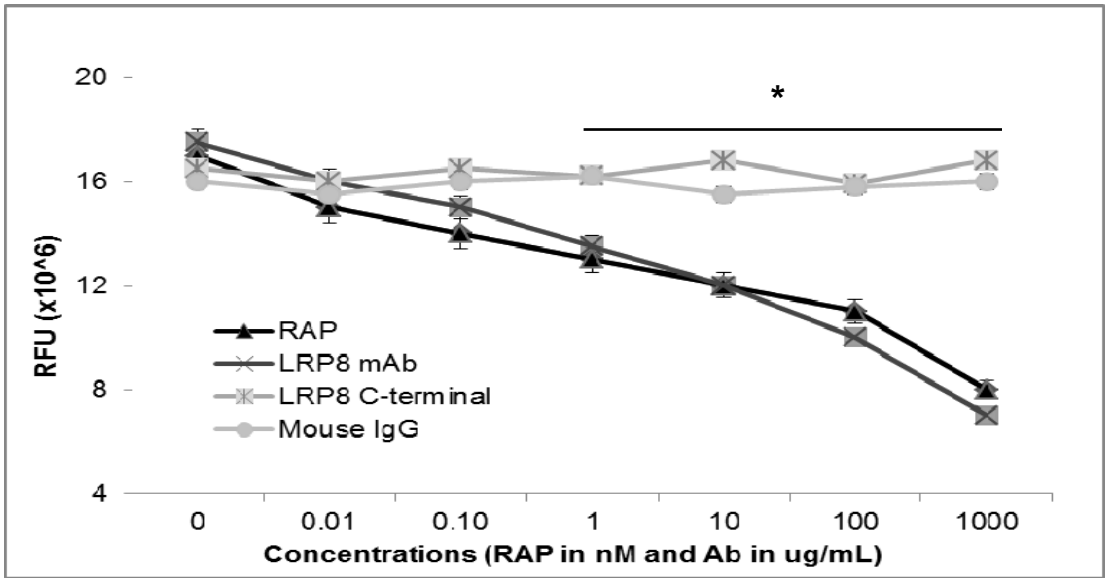

Figure 2. In a ELISA solid-phase binding assay, RAP and a monoclonal antibody targeting the ApoE4 binding region of LRP8 reduced the binding activity level between recombinant human LRP8 and ApoE4. $p<0.05$ is indicated by * in all panels.

To determine the key signaling pathways altered by LRP8 – ApoE4 signaling, ApoE4 treated cells were isolated and gene expression and reverse phase protein arrays were performed. This next set of assays was performed while animal testing protocols and approvals were being reviewed and approved for Specific Aim 3. To investigate the mechanisms controlling the robust growth effect after ApoE4 stimulation, transcriptional, proteomic, and metabolic profiling was performed using the same experimental schema described in the Methods section. Global profiling was performed in the BT-549 and MDA-MB-436 cell lines, which had the most consistent proliferative effects in response to ApoE4 stimulation as well as higher expression of LRP8 and VLDLR according to Western blotting. During serum starvation, most of the gene expression changes occurred between 8 and 48 hours of treatment, with the highest number of common probe sets occurring during the entire 48 hour treatment period. Likewise, ApoE4-induced gene expression changes were concentrated at the 48 time point, indicating that gene expression changes after 8 hours of treatment are responsible for the transcriptional changes related to serum starvation and ApoE4 stimulation (Figure 3a). Each set of triplicate clustered together with a second level of separation occurring between the group of T0/T8 and the group at T48, with this effect being more obvious with BT-549 than MDA-MB-436 (Figure 3a). For these reasons, further analyses focused on the 48 hour time point comparisons.

Serum-starvation induced the upregulation of steroid biosynthesis and lipid and amino acid metabolism pathways, while downregulating pathways associated with mitotic division and nucleotide metabolism (Figure 3b). The lipid biosynthesis network connecting the upregulated starvation-induced genes centered around SREBF2 (Figure 3d). ApoE4 stimulation increased expression of genes associated with chemokine signaling, while downregulating members of steroid biosynthesis pathways (Figure 3c and 3e). This dampening or normalization of starvation-induced steroid biosynthesis rescues TNBC cells from starvation by supplying them with an exogenous energy source mediated by ApoE4.

MAPK signaling mediates ApoE4 downstream signaling for TNBC cell proliferation

To determine the protein mediators of proliferation after ApoE4 stimulation, reverse phase protein array was performed after 48 hours of ApoE4 treatment in BT-549 and MDA-MB-436. Consistent protein changes occurred at time point 48, consistent with the results from gene expression profiling (Figure 4a). Ingenuity Pathway Analysis showed that the activated proteins were interconnected through the MAPK signaling network (Figure 4b). Proteins which were repressed after ApoE4 treatment were involved in ribosomal assembly and protein translation (Figure 4c).

To confirm the array findings, Western immunoblotting of the ApoE4 activated proteins was performed (Figure 4d). Transient and stably transfected LRP8 knockdown cell lines were established to test if ApoE4 stimulation affects TNBC cells with lower levels of ApoE's cognate receptor. The MDA-MB-231 shLRP8 stable knockdown cell line was used to establish the nude mice xenografts. LRP8 knockdown efficiency was similar in both BT-549 and MDA-MB-231 LRP8 knockdown cell lines. In BT-549 parental cells, ApoE4 increased the protein levels of phosphorylated serine-473 of histone H2AX, COX2, Rab25, and Snail. In BT-549 siLRP8 cells, this increase was reduced for phosphorylated serine-473 of histone H2AX. In BT-549 siCON and siLRP8 cells, there were no detectable levels of COX2, Rab25, or Snail. In MDA-MB-231 parental cells, there were minimal increases in phosphorylated serine-473 of histone H2AX and Snail after ApoE4 treatment. In MDA-MB-231 shLRP8 cells, both proteins were slightly reduced in comparison to shCON.

Metabolomic profiling – alternative metabolic pathways during serum starvation and the coordination of LRP8/ApoE4 signaling to rescue cells from cellular stress

BT-549 cells were treated with ApoE4 for 48 hours and GC/MS and LC/MS/MS profiling platforms were used by Metabolon, Inc. to determine the metabolic biochemical changes that mediate growth and survival of ApoE4 activated cells. Three comparisons were focused upon, the effects of serum-starvation (serum-free cells at 48 hours / regular media cells at 48 hours), the rescue effects of ApoE4 treatment (ApoE4 treated cells at 48 hours / serum-free cells at 48 hours), and the rescue effects potentially reversed by LRP8 knockdown (siLRP8+ApoE4 at 48 hours / siCON at 48 hours). Serum-starvation resulted in the activation of Warburg metabolism, with increases in tryptophan and reduced NAD+ levels. Glycolysis intermediates, such as fructose-6-phosphate and 3-phosphoglycerate, were increased in serum-starved cells. Reduced glutathione and hydroxycholesterol metabolites generated by reactive oxygen species were also elevated.

In response to ApoE4 treatment, the cells had decreased levels of the previously activated glycolysis intermediates and reduced levels of some oxidative stress chemicals and cholesterol metabolites. In response to LRP8 knockdown, cells reversed some of the rescue effects and reconstituted levels of key chemicals in nucleic acid metabolism and lipid metabolites.

Figure 3

a.

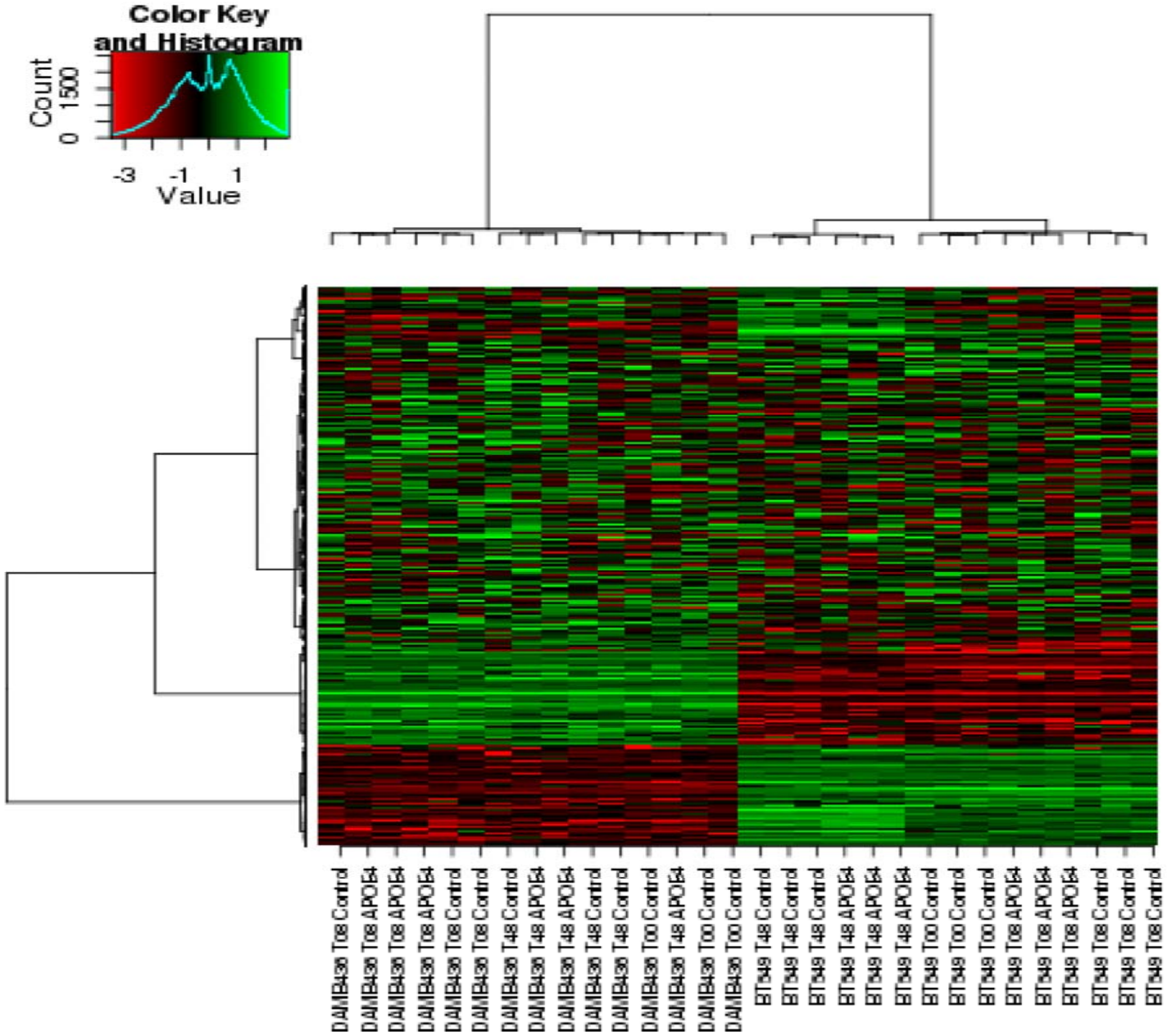
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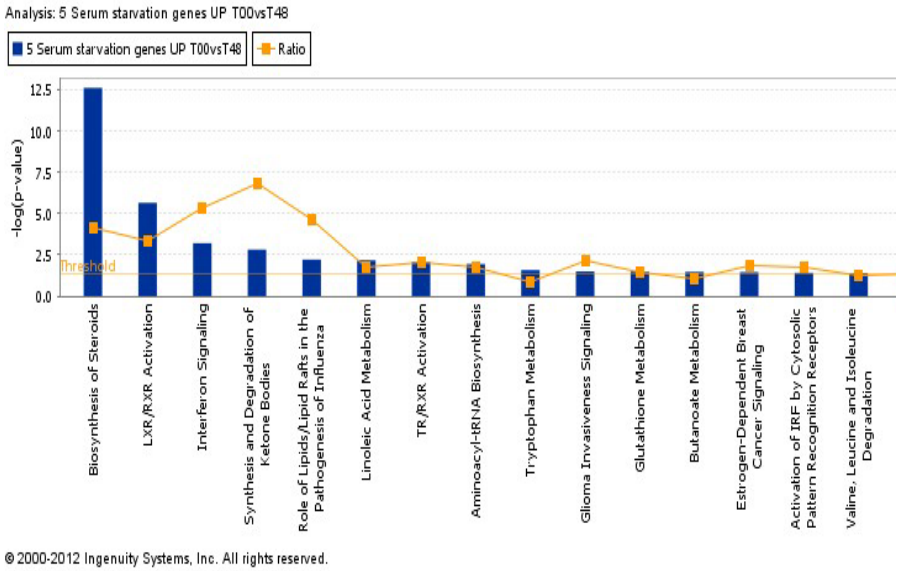
Figure 3. Modulation of steroid and lipid biosynthesis canonical pathways during serum-starvation and after ApoE4 stimulation

b. Upregulated pathways after serum-starvation



c. Downregulated pathways after ApoE4 stimulation

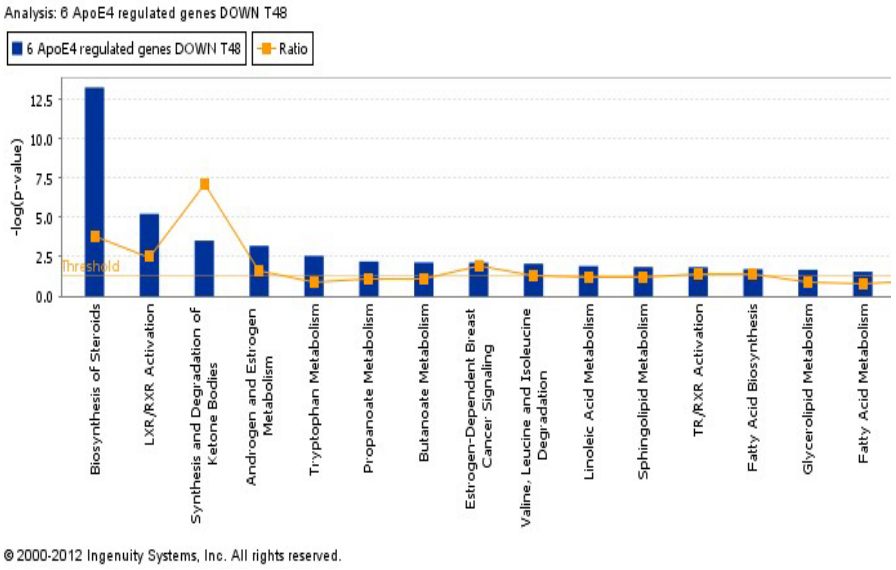
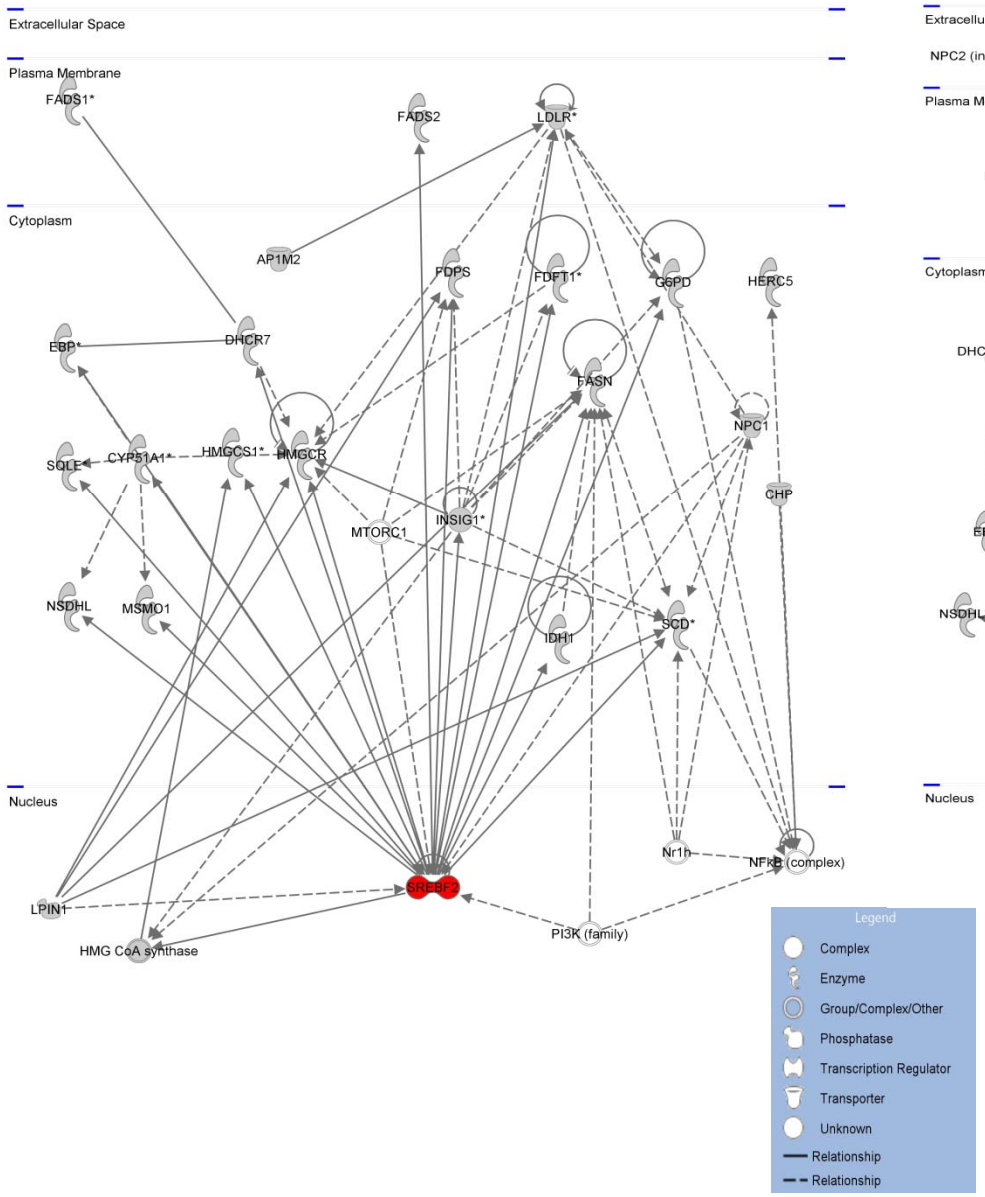


Figure 3. Modulation of lipid biosynthesis network during serum-starvation and after ApoE4 stimulation

d. Upregulated network after serum-starvation



e. Downregulated network after ApoE4 stimulation

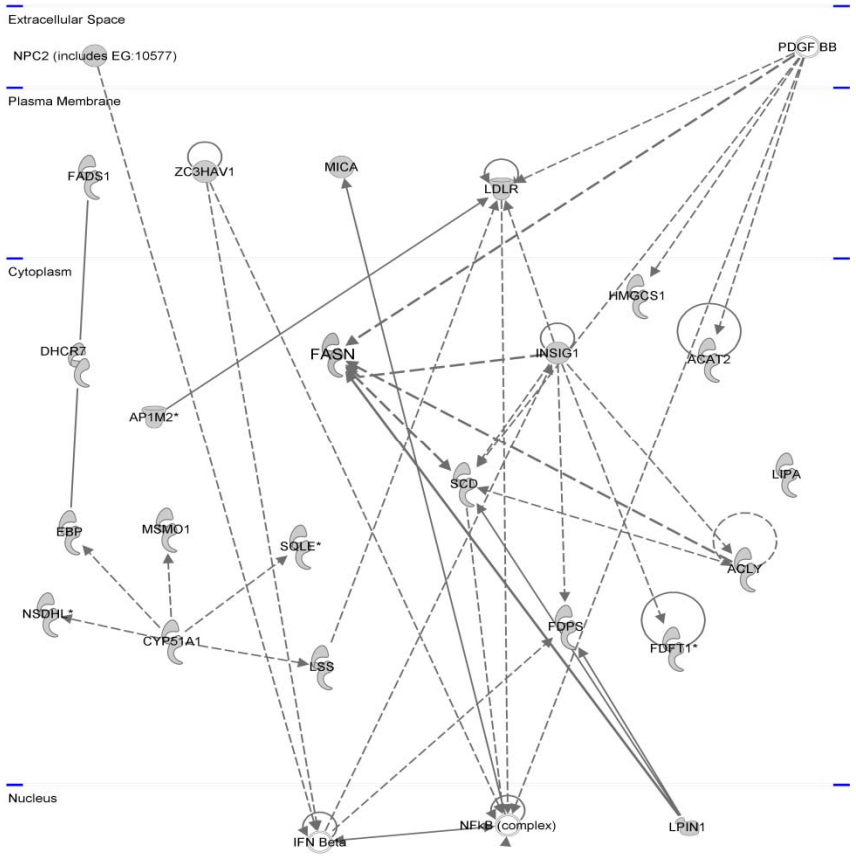
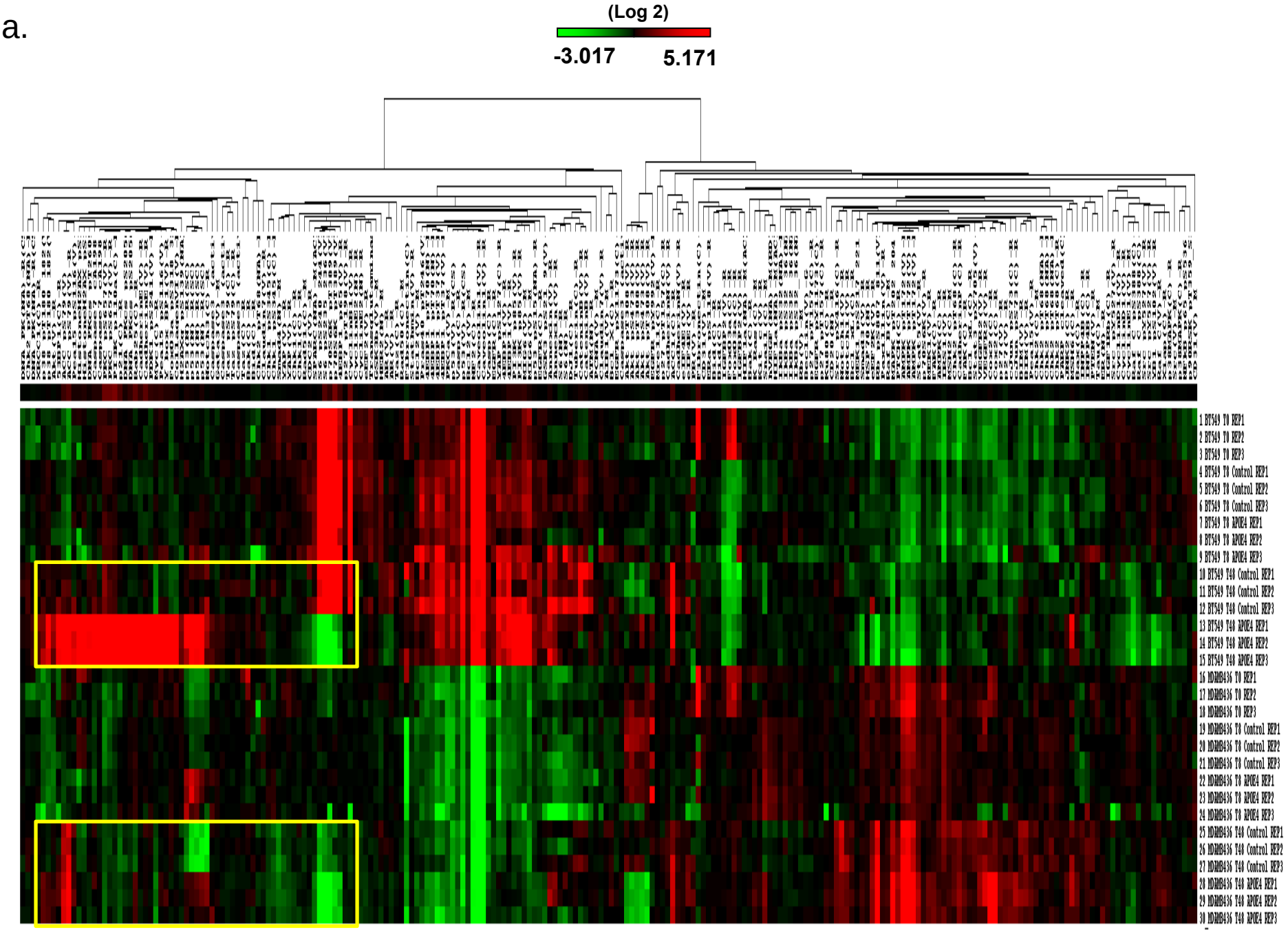


Figure 4

a.


(Log 2)
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5.171
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4 BT549 T0 Control REP1
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10 BT549 T48 Control REP1
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14 BT549 T48 APOE4 REP2
15 BT549 T48 APOE4 REP3
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18 MDAMB436 T0 REP3
19 MDAMB436 T0 Control REP1
20 MDAMB436 T0 Control REP2
21 MDAMB436 T0 Control REP3
22 MDAMB436 T0 APOE4 REP1
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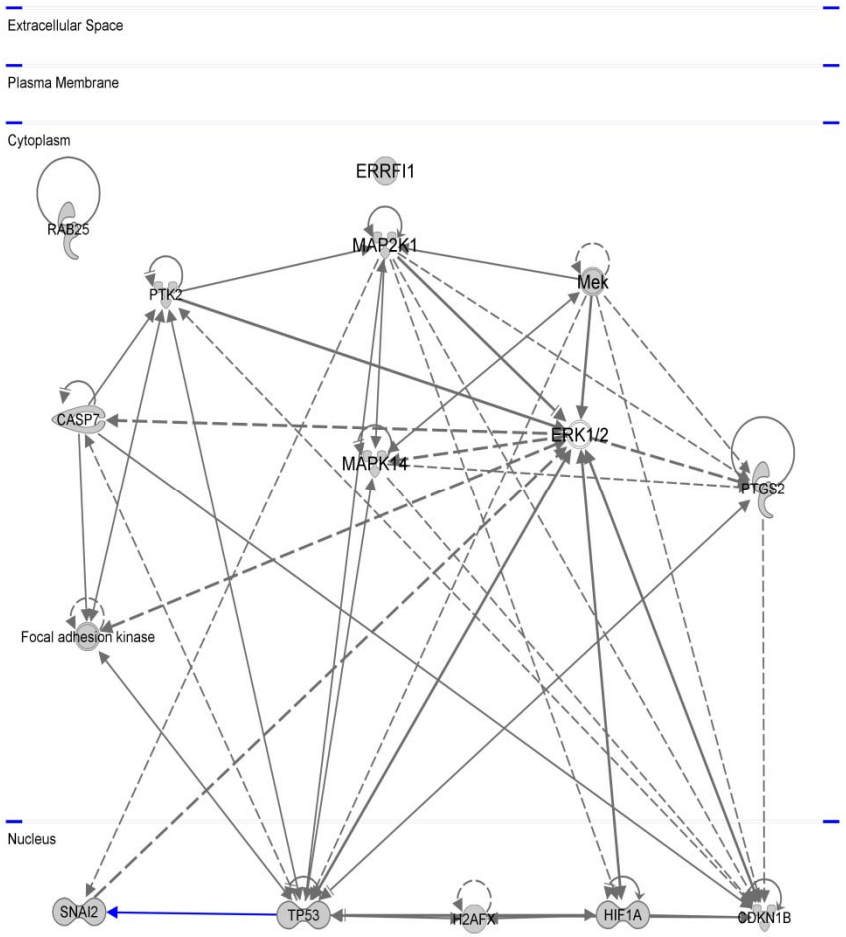
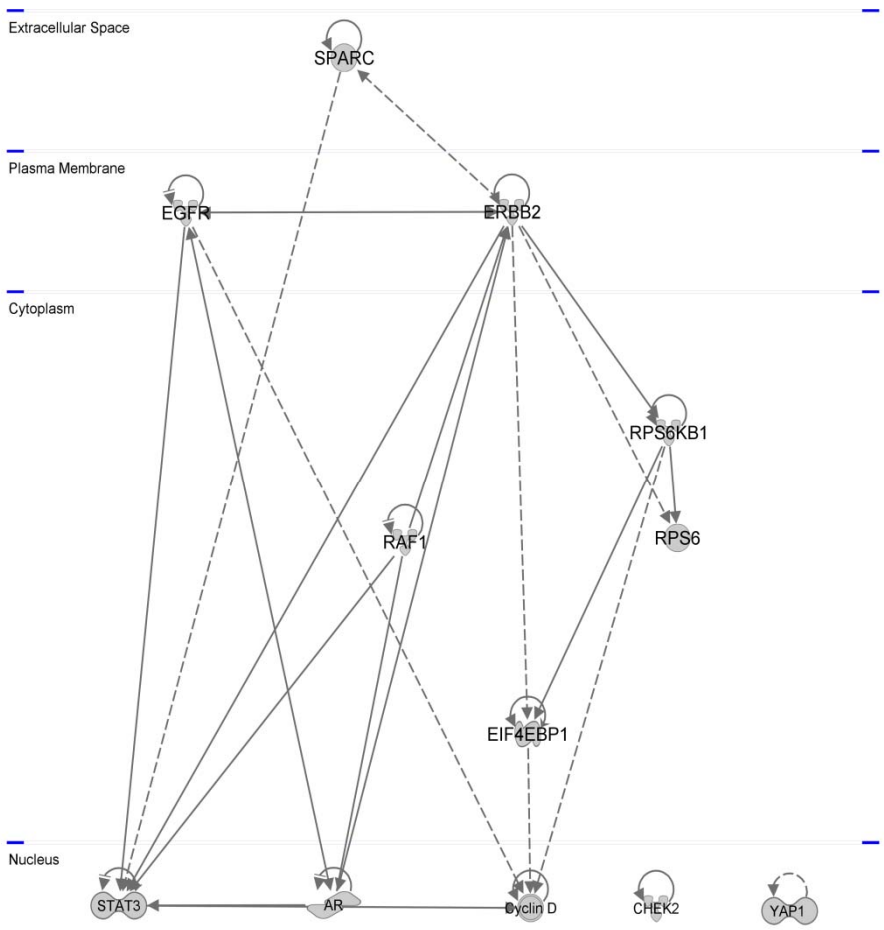
27 MDAMB436 T48 Control REP3
28 MDAMB436 T48 APOE4 REP1
29 MDAMB436 T48 APOE4 REP2
30 MDAMB436 T48 APOE4 REP3

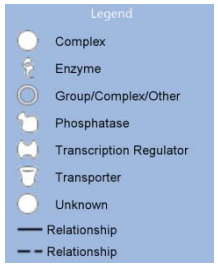
Figure 4. ApoE4 regulated proteins control cellular proliferation and cell cycle control

b. Upregulated proteins after ApoE4 stimulation



c. Downregulated proteins after ApoE4 stimulation





Specific Aim 3: To determine whether silencing critical growth genes effects the growth of triple-negative breast cancer xenografts

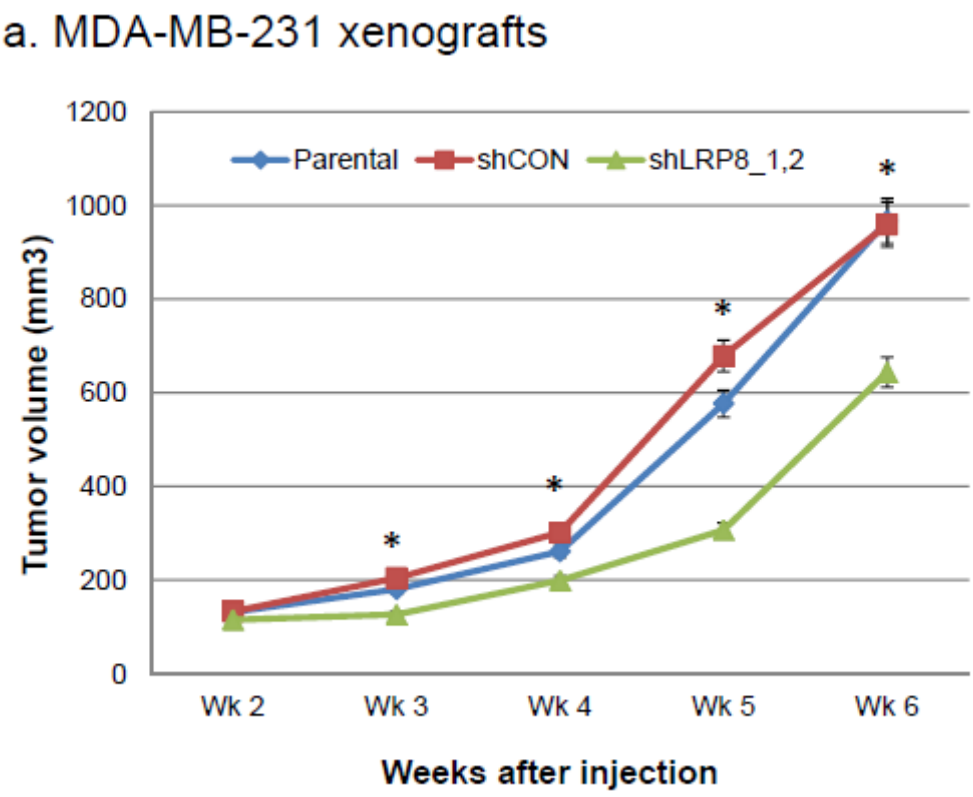
Task 6: Obtain Institutional Animal Care and Use Committee Protocol Approval (Office of Research Administration), Category 2 Protocol (little or no pain or distress protocol), import from The Jackson Laboratory (Stock: 002019, Strain: NU/J, Genotype: *A/A Tyrc/Tyrc Foxn1nu/Foxn1nu*), total 35 mice/experiment, 10 mice/group, 3 groups (shTARGET, shCONTROL, uninjected), total 3 experiments, 6 months/experiment.

Task 7: Establish orthotopic xenografts using MDA-MB-231 TNBC cells.

Task 8: In vivo gene silencing of target gene with shRNA delivery into the TNBC xenografts

Task 9: Examination of orthotopic xenograft specimens and major organs (order animals for repetitions of the above experiment)

Results: Above approvals were obtained, as evidenced by the following results of the xenograft studies. Preliminary results indicate that shLRP8 xenografts have reduced tumor volume, in comparison to parental or shCON MDA-MB-231 xenografts. Another replicate of this experiment is pending, as well as histological tissue examination and protein assays.



KEY RESEARCH ACCOMPLISHMENTS

- Identification of the LRP8 – ApoE4 signaling axis in the proliferation of TNBC cell lines

- Identification of altered fatty acid and glycolytic pathways in response to LRP8 – ApoE4 signaling in TNBC cell lines

- Preliminary shLRP8 xenograft results indicate that *in vivo* depletion of LRP8 reduces tumor volume (pending final results)

REPORTABLE OUTCOMES

- Manuscript is being compiled in anticipation of publication in 2013

CONCLUSION

The summarized work reveals potential new therapeutic targets for a subset of breast cancers which have limited therapeutic options. LRP8 and VLDLR are two surface membrane receptors that act as growth signals for triple-negative breast cancer may be targeted with targeted therapeutics. In response to LRP8 – ApoE4 signaling, downstream pathways are activated that result in altered metabolic adaptations which allow TNBC cells to grow in the absence of nutrients.

REFERENCES

1. Hess KR, Anderson K, Symmans WF, Valero V, Ibrahim N, Mejia JA, Booser D, Theriault RL, Buzdar AU, Dempsey PJ, Rouzier R, Sneige N, Ross JS, Vidaurre T, Gomez HL, Hortobagyi GN, Pusztai L. Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer. J Clin Oncol 24:4236-44, 2006.
2. Peintinger F, Anderson K, Mazouni C, Kuerer HM, Lin F, Hortobagyi GN, Symmans WF, Pusztai L. Thirty-gene pharmacogenomic test correlates with residual cancer burden after preoperative chemotherapy breast cancer. Clin Cancer Res 13(14):4078-82, 2007.

APPENDICES

- None